Acute Versus Chronic Effects of Whey Proteins on Calcium Absorption in Growing Rats

YONGDONG ZHAO,* BERDINE R. MARTIN,* MERYL E. WASTNEY,† LINDA SCHOLLUM,‡ AND CONNIE M. WEAVER*,[1]

Foods and Nutrition Department, Purdue University, West Lafayette, Indiana 47907; †Metabolic Modeling Services, Hamilton, New Zealand; and ‡Fonterra Research Centre, Palmerston North, New Zealand

The acute and chronic effects of whey proteins on calcium metabolism and bone were evaluated. In acute studies, 8-week-old male rats were gavaged with 50 mg whey protein concentrate (WPC) and 25 mg calcium. ^{45}Ca was administered intravenously or orally. Kinetic studies were performed, and femurs were harvested. Four of seven WPCs significantly increased femur uptake of ^{45}Ca compared with controls. One WPC at 50 mg enhanced calcium absorption over a range of calcium intakes from 35.1 ± 9.4% to 42.4 ± 14.0% ($P < 0.01$). Three of the most effective WPCs were tested further in a chronic feeding study. One hundred 3-week-old rats were randomly divided into four adequate dietary calcium (ADC; 0.4% Ca) groups (control of 20% casein and three WPC groups with 1% substitution of casein with each of three WPCs) and two low calcium (LC; 0.2% Ca) groups (control of 20% casein and one WPC group with 1% substitution of casein with one WPC). After 8 weeks, there was no effect of WPCs on femur uptake of ^{45}Ca among ADC groups and there was no effect of WPCs on calcium retention, femur breaking force, femur bone mineral density, or total femur calcium at either dietary calcium intake. However, whole body bone mineral content (BMC) was significantly higher ($P < 0.05$) in the three whey protein concentrate ADC groups compared with the ADC control group. Total BMC at the proximal tibia in whey protein ADC groups was increased, as shown by peripheral quantitative computed tomography. Our results indicate that the acute calcium absorption–enhancing effect of whey proteins did not persist through long-term feeding in rats. However, the initial enhancement of calcium absorption by whey protein was sufficient to increase BMC. Exp Biol Med 230:536–542, 2005

Key words: whey protein; calcium retention; absorption; kinetics

Introduction

Whey proteins comprise about 20% of total bovine milk proteins and have gained popularity as functional foods. Two whey proteins, α-lactalbumin and glycomacropeptide (GMP), are of interest in improving bone health. α-Lactalbumin is thought to enhance calcium use (1). This protein has calcium-binding sites that may prevent calcium from precipitation in the intestine. The chymosin digest product from the C-terminal end of k-casein, GMP, has been shown to enhance calcium retention and to decrease bone resorption quantified by urinary ^{3}H-tetracycline in both growing and ovariectomized rats (2). However, most studies (3–8) tested whey protein concentrates (WPCs), which contain many different types of whey proteins. Different whey protein concentrates have distinct spectra of proteins, depending on the preparation and whey sources. Whey protein concentrates rich in GMP may benefit bone health through promoting calcium absorption or retention. Furthermore, 1% or 2% WPC in the diet has been associated with increased breaking energies of the bones in ovariectomized rats (3). The mechanism for improved mechanical properties is not clear because of no change in calcium balance. In a follow-up study (4), heat-stable whey protein, low-molecular-weight whey protein (<24,500), and ethanol-precipitated whey protein were obtained from WPCs. Diets containing 1% of each protein fraction increased the total amounts of amino acids and proline in the femur, which may have been responsible for the increased femur strength. Two clinical studies observed that the basic fraction of whey protein (milk basic protein) increased bone formation and suppressed bone resorption in both adult women (5) and adult men (6), manifested by bone biomarkers. Our studies were designed to investigate the acute and chronic impacts of various WPC preparations on bone and calcium metabolism in growing rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan Inc., Indianapolis, IN) were housed individually in stainless steel

This study was supported by funding from New Zealand Milk Ltd., and whey protein concentrates were provided by the Fonterra Research Centre.

[1] To whom correspondence should be addressed at Department of Foods and Nutrition, Purdue University, 700 W. State Street, West Lafayette, IN 47907-2059. E-mail: weavercm@purdue.edu

Received April 8, 2005.
Accepted May 15, 2005.

1535-3702/05/2308-0536$15.00

cages with *ad libitum* access to deionized water and diets. Animals were exposed to a 12-hr light:dark cycle. Room temperature and humidity were maintained constant during the study. All of the animal experiments were approved by the Purdue Animal Care and Use Committee.

Experiment Design. *Preliminary Studies.* Eight-week-old rats were adapted to an AIN93G diet (7) (Dyets Inc., Bethlehem, PA) for 1–2 weeks before they were implanted with jugular catheters (0.51 mm i.d., 0.94 mm o.d.; VWR, Chicago, IL) under anesthesia (ketamine:xylazine 90:10 mg/kg). Detailed surgery procedure is described elsewhere (8). Animals were stabilized using presurgery weight as a gauge. After a 2-day recovery, rats were fasted for at least 8 hrs and received an oral dose containing calcium acetate (Sigma Inc., St. Louis, MO) and WPC; ^{45}Ca (5–50 mCi/mg Ca; Amersham Biosciences, Piscataway, NJ) was given either orally (20 μCi) or intravenously (10 μCi). Blood samples (0.3 ml) were collected at 0, 5, 10, 15, 20, 30, 40, 60, 80, 130, 180, 240, and 300 mins after dosing, and serum was obtained for ^{45}Ca assay. Total blood loss was replaced with saline:dextrose (1:1) during sampling. Rats were killed 24 hrs after ^{45}Ca administration, and femurs were harvested for ^{45}Ca analysis. These procedures were used in all three acute studies unless stated otherwise.

Study 1: Comparison of WPCs. Seven different whey protein products were tested for their Ca absorption–enhancing effect with varying protein compositions. A detailed composition of each WPC is listed in Table 1. The protein and calcium contents of these WPCs ranged from 770 to 870 and 0.3 to 4.7 g/kg, respectively. At the dose (50 mg) we tested, WPCs contained less than 0.25 mg Ca, which was negligible compared with the calcium load (25 mg) used. Whey protein concentrate-1, WPC-2, WPC-9, and WPC-10 were processed from cheese whey, whereas WPC-5 and WPC-7 were from mineral acid whey. Whey protein concentrate-1, WPC-9, and WPC-10 were WPCs containing GMP, WPC-2 and WPC-8 were whey protein products with enriched levels of GMP, and WPC-5 and WPC-7 were whey protein products that did not contain GMP. All WPCs were provided by Fonterra Research Centre (Palmerston North, New Zealand). In this study, animals ($n = 7$–14/WPC group, $n = 30$ in control) were gavaged with 25 mg calcium with or without 50 mg of the different WPCs.

Study 2: Dose-Response Study. A dose-response effect of WPC-1 on calcium absorption was performed by giving 0, 25, 50, or 75 mg of WPC-1 mixed with 25 mg calcium to the rats. Six or seven rats per group received ^{45}Ca orally, whereas three rats per group received ^{45}Ca intravenously.

Study 3: WPC and Calcium Interaction. The interaction between different calcium loads and WPC was evaluated by giving 10, 50, or 75 mg calcium with or without 50 mg of WPC-1 to animals. Five to seven rats per group received ^{45}Ca orally, whereas three rats per group received ^{45}Ca intravenously.

Chronic Study. One hundred 3-week-old weanling rats were fed a modified AIN93G diet for a week before they were randomly assigned to six groups and were continued on experimental diets for 8 weeks. Control (20% casein), WPC-1 (1%), WPC-5 (1%), and WPC-10 (1%) in a calcium adequate (ADC; 0.4% Ca, $n = 16$/group) AIN93G base or control and WPC-10 in a low calcium (LC; 0.2% Ca, $n = 18$/group) AIN93G base were tested. The three WPCs chosen for the chronic study were shown to be the most effective in enhancing calcium absorption acutely. Calcium in the diet was provided as calcium carbonate. Based on the results from acute studies, 50 mg of WPC had a greater enhancing effect on calcium absorption. One percent WPC in the diet provides approximately 50 mg WPC per meal, assuming a daily intake of 15 g diet in the rats. All the diets were isonitrogenous and isocaloric. The diet composition is described in Table 2.

A 3-day calcium balance study was performed during the first week of feeding the experimental diets. Food intake and body weight were measured twice a week throughout the experiment. Whole body dual x-ray energy absorptiometry (DXA; IQ-100; Lunar Inc., Madison, WI) was performed on all the rats after 7 weeks of feeding.

After 8 weeks of feeding, 4-day metabolic balance studies were performed on all the rats, and ^{45}Ca kinetics were measured in a subset of nine rats in each ADC group. Procedures for ^{45}Ca kinetics were as described for the acute studies: ^{45}Ca was administered either orally ($n = 6$–7/group) or intravenously ($n = 2$–3/group) while 50 mg of the respective WPCs and 25 mg calcium were gavaged. Rats were placed in metabolic cages beginning the day of isotope

Table 1. Composition of WPCs Expressed as Percent of Powder Weight

WPC no.	β-Lactoglobulin	α-Lactalbumin	Bovine serum albumin	IgG	GMP	Other[a]	Calcium	Moisture/ash/ lactose
1	39	10	3	3	17	8	0.31	20
2	2	1	≤1	≤1	81	10	0.03	6
5	47	13	2	3	1	14	0.15	20
7	56	23	3	4	≤1	8	0.26	6
8	2	3	≤1	≤1	78	11	0.47	6
9	37	8	2	4	17	12	0.32	20
10	43	20	1	4	20	6	0.21	6

[a] Other, minor proteins and peptides, etc.

Table 2. Diet Composition During Chronic Feeding Study[a]

Groups[2]	Calcium content (wt/wt)	Protein composition (wt/wt)
Control ADC	0.4%	20% casein
WPC-1 ADC	0.4%	19% casein + 1% WPC-1
WPC-5 ADC	0.4%	19% casein + 1% WPC-5
WPC-10 ADC	0.4%	19% casein + 1% WPC-10
Control LC	0.2%	20% casein
WPC-10 LC	0.2%	19% casein + 1% WPC-10

[a] Diets were modified based on AIN 93G: 1000-g diet contained 397.5 g cornstarch, 200 g protein (casein or mixture of casein and WPC), 132 g dextrinized cornstarch, 90 g sucrose, 70 g soybean oil, 50 g fiber, 35 g Mineral Mix minus Calcium (93G-MX), 10 g vitamin Mix 93-VX, 3 g L-cystine, and 2.5 g choline bitartrate. Calcium carbonate was either 10 g in ADC groups or 5 g in LC groups.

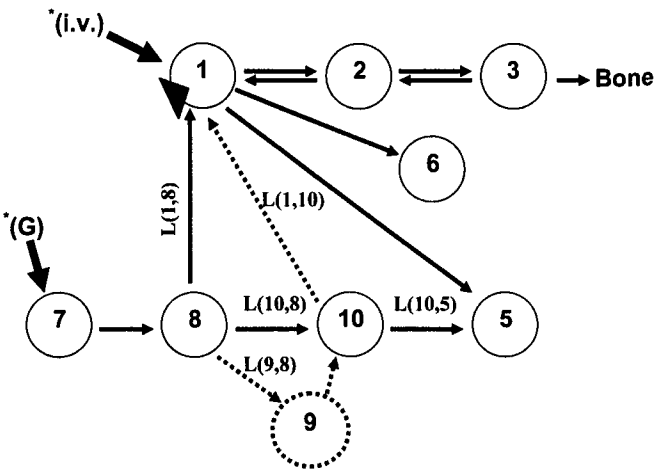

Figure 1. Kinetic models for calcium absorption in rats. Circles represent compartments. Compartment 1, blood; Compartments 2 and 3, exchangeable calcium pools; Compartment 5, feces; Compartment 6, urine; Compartment 7, stomach; Compartment 8, upper small intestine; Compartment 9, delay compartment in higher Ca load groups; Compartment 10, lower small intestine. Triangle indicates the sampling compartment (blood). Arrows represent movement of calcium between compartments. Dash lines were pathways required for high calcium loads. L(I,J) is the fractional transfer coefficient (/min) of ^{45}Ca from Compartment J to Compartment I. Tracer (^{45}Ca) was administered to either compartment 7 (orally or by gavage) or Compartment 1 (intravenously). At low Ca loads (10 and 25 mg), $L(9,8) = 0$ and $L(1,10) = 0$, whereas at high Ca loads (50 and 75 mg), $L(10,8) = 0$.

administration and continuing for 4 days. Rats were killed after the metabolic balance period. Both femurs and left tibias were collected. Femurs were stored in saline under 4°C for mechanical testing, DXA scan, and ^{45}Ca analysis. Tibias were wrapped with 10% formalin-soaked gauze and frozen at –80°C, placed in 70% ethanol, and stored at 4°C 1 week before being scanned by peripheral quantitative computed tomography (pQCT).

Femur and Serum ^{45}Ca Analysis. Femurs were dissolved in 3 ml concentrated nitric acid overnight and diluted to 25 ml. From this, ^{45}Ca in a 1-ml aliquot was counted in a liquid scintillation system (LS 6500; Beckman Coulter Inc., Fullerton, CA). Serum samples (100 µl) were bleached by 100 µl H_2O_2, 25 µl HCl, and 25 µl KOH before liquid scintillation counting.

Kinetic Modeling of ^{45}Ca Absorption. WinSAAM (Simulation Analysis and Modeling) program and a multicompartmental calcium model were employed to calculate calcium absorption efficiency from serum ^{45}Ca kinetic data obtained in each group as previously described (9, 10). Serum ^{45}Ca data obtained from rats given intravenous ^{45}Ca administration in each group were fitted by the model (Fig. 1) to determine ^{45}Ca transfer rates among Compartments 1, 2, and 3 and bone. Loss into urine and feces were taken into account by fixing them to those generated from similar aged rats (11). Then serum ^{45}Ca data from rats given ^{45}Ca orally were fitted by the model using fractional transfer rates established from the intravenous group. In our previous studies (8–11), calcium loads were ≤25 mg and one absorption compartment was enough to fit the serum ^{45}Ca data. Interestingly, when 50 or 75 mg calcium was gavaged, ^{45}Ca profiles were different from those when ≤25 mg Ca was gavaged. In most cases, a quick but low serum ^{45}Ca peak was followed by a slower but high serum ^{45}Ca peak, which indicated the existence of two calcium absorption sites at high calcium loads. The data could only be fitted by adding a second absorption Compartment 10 to the model with a delay Compartment 9 between the two absorption sites (Compartments 8 and 10). Calcium absorption was calculated as Eqs. 1 and 2 for low and high calcium loads, respectively.

In the 10- and 25-mg calcium groups, there is only one absorption compartment (Compartment 8) where calcium flows directly from Compartment 8 to Compartment 10. Calcium absorption was calculated as the ratio of calcium moving from intestine into blood versus calcium moving out of intestine.

$$\text{Ca absorption } (\%) = \frac{L(1,8)}{L(1,8)+L(10,8)} \times 100 \qquad (1)$$

In the 50- and 75-mg calcium groups, a delay compartment, Compartment 9, was added to the model, and calcium flows from Compartment 8 to Compartment 10 through Compartment 9. Calcium absorption was calculated as the sum of absorption from both absorption compartments (Compartments 8 and 10). The first part of the equation represents fractional calcium absorption from Compartment 8, and the second part of the equation represents fractional calcium absorption from Compartment 10.

$$\text{Ca absorption } (\%) = \left[\frac{L(1,8)}{L(1,8)+L(9,8)} + \frac{L(1,10)}{L(1,10)+L(5,10)} \times \frac{L(9,8)}{L(1,8)+L(9,8)}\right] \times 100 \qquad (2)$$

where L(I,J) is calcium transfer rate from Compartment J to Compartment I, i.e., $L(1,8)$ is calcium transfer rate from Compartment 8 to Compartment 1.

Calcium Balance. The amount of calcium intake during the balance studies was calculated as the product of food intake and dietary calcium concentration plus calcium gavaged. Dried feces were ashed at 600°C for 3 days. The ash was dissolved in a few drops of 50% (wt/wt) concentrated HCl and diluted with 0.5 m*M* $LaCl_3$. Total calcium in urine and feces were analyzed by atomic absorption spectrometry (A Analyst 300; Perkin Elmer, Inc., Norwalk, CT).

$$\text{Calcium apparent absorption (\%)} = (\text{Ca intake} - \text{Fecal Ca}) \times 100/\text{Ca intake}$$

$$\text{Calcium retention rate (\%)} = (\text{Ca intake} - \text{Urinary Ca} - \text{Fecal Ca}) \times 100/\text{Ca intake}$$

Femur Analysis. Left femurs from the chronic study were measured for length, width, wet weight, and density by underwater weighing. The left femurs were broken in a three-point bending test on a TA-XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) using a test speed of 1 mm/sec. Data were expressed as peak force (kg). Right femurs were scanned by DXA (IQ-100; Lunar Inc., Madison, WI). Bone mineral density (BMD) and bone mineral content (BMC) of the proximal region, midshaft, and distal regions were reported. The right femurs were dissolved in 3 ml concentrated nitric acid overnight and analyzed for ^{45}Ca radioactivity and total calcium.

Tibia pQCT Measurements. Cross-sectional sites 1.0 mm thick on the bones were scanned by pQCT (model Research SA+ of Norland Stratec XCT; Stratec Electronics, Pforzheim, Germany), using a 0.46-mm collimation (4 × 105 counts/s) and a 0.08-mm voxel size. Tomographs generated by pQCT were analyzed with software version 5.40 (Norland Stratec Medizintechinik GmbH, Birkenfeld, Germany). Each tibia was scanned at the midshaft (50% of total length from proximal end) and proximal metaphysis (12% of total length from proximal end). Corresponding parameters are BMC, BMD, and cross-sectional area (CSA). To separate soft tissue from bone and trabecular from cortical bone, the thresholds of 500 and 900 mg/cm^3 were used, respectively. Separation was performed by use of the software's contour mode 1, peel mode 2.

Statistical Analysis. Data were analyzed using the SAS statistical program (version 8.0; SAS Institute, Cary, NC). Data are expressed as means ± SD. Main effects were analyzed by the general linear models procedure. Comparisons of multiple group means were performed using Fisher's least significant difference test. Differences were considered significant when $P < 0.05$. Comparisons between two group means were performed by independent *t* tests. Whole body BMD and BMC were analyzed using body weight as a covariate.

Results

Preliminary Study 1: Comparison of WPCs. Fifty milligrams of WPC-1, WPC-8, WPC-9, and WPC-10

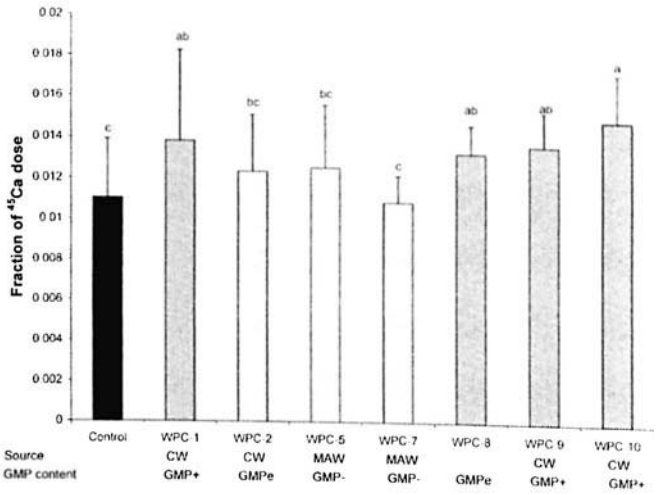

Figure 2. Femur uptake of ^{45}Ca expressed as fraction of dose from different WPC treatments compared with control. Values are means ± SD. Different letters indicate significant differences between groups ($P < 0.05$). $n = 7$–14/WPC group; $n = 30$ in control group. CW, cheese whey; MAW, mineral acid whey; GMP+, contain low amount of GMP; GMP–, contain no GMP; GMPe, enriched with high levels of GMP.

significantly increased femur uptake of ^{45}Ca with a 25-mg calcium load (Fig. 2). Serum ^{45}Ca kinetic curves were consistent with femur uptake results (Fig. 3). Serum ^{45}Ca peak concentration from WPC groups was higher than control except from WPC-7. Three of them, WPC-10, WPC-1, and WPC-5, had the most pronounced effect.

Preliminary Study 2: Dose-Response Study. Both 25 and 50 mg of WPC-1 increased calcium absorption. However, a higher amount (75 mg) of WPC-1 did not enhance calcium absorption (Fig. 4). Calcium absorption results determined by kinetic analysis were consistent with, but more discriminative than, femur uptake of ^{45}Ca (Fig. 4).

Preliminary Study 3: WPC and Calcium Interaction. The increase of percent calcium absorption induced by 50 mg of WPC-1 was less pronounced at higher calcium levels (Table 3). Because of the small sample size

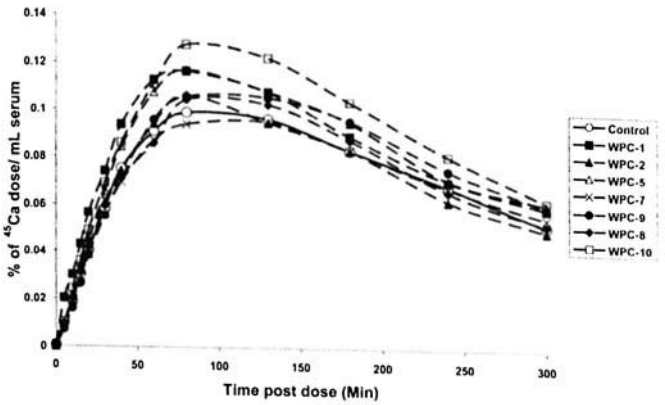

Figure 3. Serum ^{45}Ca kinetics of different WPC treatment groups and control within 5-hr postdose. Group means of serum ^{45}Ca concentration were plotted against time postdose. Solid line represents control group. Dash lines represent WPC groups.

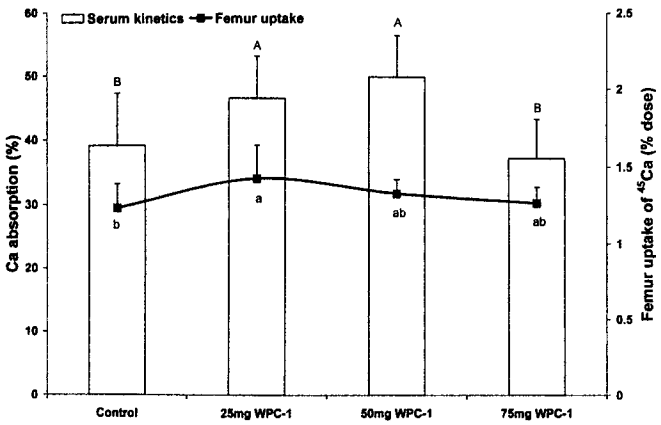

Figure 4. Dose-response effect of WPC-1 on calcium absorption and femur ^{45}Ca uptake at 25 mg calcium level. Percent calcium absorption was calculated from serum ^{45}Ca kinetics in each group. Femur ^{45}Ca uptake was expressed as percent of dose/femur. Values are means ± SD. Different letters indicate significant differences between groups ($P < 0.05$).

(n = 5–7/group), differences within calcium load did not achieve statistical significance. When the data were pooled by treatment with calcium load as a block factor, 50 mg of WPC-1 significantly increased calcium absorption from 35.1 ± 9.4% to 42.4 ± 14.0% ($P < 0.01$; Table 3).

Chronic Study. Feed efficiency only increased in one group (WPC-1; Table 4). WPC-1 increased apparent calcium absorption from 87.3 ± 6.2% to 90.0 ± 4.0% during the first week of feeding (Table 4). Low calcium groups had higher calcium absorption and retention rates than adequate calcium groups at both 1 and 8 weeks. There was no effect of WPC feeding on apparent calcium absorption, calcium retention rate, or urinary calcium excretion at either calcium intake level (Table 4). Among the four ADC groups, there were no differences in femur uptake of ^{45}Ca (data not shown). Serum ^{45}Ca curves were almost identical (data not shown). Nor were there differences in femur weight, femur length, femur width, femur density, femur breaking strength, femur BMD, or femur BMC at the proximal, distal, or midshaft regions between WPC treatment groups and controls at either calcium intake level (data not shown). Addition of WPC-1, WPC-5, or WPC-10 at 1% significantly increased whole body BMC by DXA after 8 weeks of feeding at an adequate calcium intake ($P<0.05$; Fig. 5A). WPC-10 did not show any improvement on whole body BMC at low calcium intake. Whole body BMD was not different between WPC and control groups at each calcium intake (data not shown). Peripheral QCT showed an increase of total BMC in the proximal tibia by the addition of WPCs at adequate calcium intake (Fig. 5B). Whey protein concentrate-5 significantly increased trabecular BMC (Fig. 5C). There was no effect of WPC on vBMD or CSA in the proximal tibia. Midshaft pQCT parameters were not changed by WPC at either calcium intake (data not shown).

Table 3. Acute Effect of 50 mg WPC-1 on Percent Calcium Absorption (Kinetic Analysis) at Various Calcium Loads[a]

	% calcium absorption		
	Control	50 mg WPC-1	Difference between groups
10 mg Ca	47.5 ± 5.6 (5)	56.2 ± 11.3* (7)	8.7
50 mg Ca	33.3 ± 3.3 (6)	39.8 ± 9.7 (6)	6.5
75 mg Ca	26.6 ± 1.6 (6)	30.9 ± 5.3* (7)	4.3
Pooled data	35.1 ± 9.4 (17)	42.4 ± 14.0** (20)	7.3

[a] Values are means ± SD (n).
*$P = 0.07$; **$P < 0.01$ compared with control.

Discussion

Whey protein concentrates include multiple calcium-binding proteins. Depending on the whey source and preparation procedure, the composition of WPCs may vary greatly. Glycomacropeptide has been patented by Nestle Inc. for its ability to enhance calcium retention and inhibit bone resorption (2). We evaluated a variety of whey proteins produced by different processes for their abilities to enhance calcium absorption. In our preliminary study 1, most of the WPCs that contained GMP (i.e., WPC-1, -2, -8, -9, and -10) significantly increased calcium absorption. However, the response of ^{45}Ca use to WPCs was not dependent on GMP, because WPC with relatively low GMP (WPC-10) elicited a greater increase of calcium use than the products enriched with higher levels of GMP (WPC-2 and -8). It is possible that there are other yet unidentified components in WPCs that also stimulate calcium absorption. Further fractionation of WPCs is warranted to identify individual calcium absorption enhancers other than GMP. It was also clear that the enhancing effect of WPCs was not simply caused by the meal effect, because one WPC containing no GMP (WPC-7) did not have any influence on calcium absorption at all. We did not observe a linear, but rather a biphasic, dose-response effect of WPCs on calcium absorption, indicating that calcium bound to whey proteins may limit calcium absorption. This phenomenon has been reported for higher concentrations of casein phosphopeptide. Casein phosphopeptide can chelate calcium and prevent the precipitation of calcium salts. It reduced calcium absorption when given at ≥20% of a meal (11).

Interestingly, we found two different calcium absorption patterns when rats were given a high (50 and 75 mg) or a low (10 and 25 mg) bolus dose of calcium. We postulated that calcium absorption was saturated quickly in the upper small intestine (especially duodenum) with high calcium loads. It is very likely that a portion of calcium might have been precipitated in the intestine as serum ^{45}Ca reached the plateau after the first peak. When the unabsorbed calcium reached the cecum and colon, bacteria that produce short chain fatty acids could resolubilize the calcium (13). Subsequent calcium absorption would be reflected as a second serum ^{45}Ca peak.

Table 4. *Body Weight, Food Intake, Feed Efficiency, and Calcium Metabolism Parameters from Chronic Study During 8-Week Feeding*[a]

Variable[b]	Control ADC	WPC-1 ADC	WPC-5 ADC	WPC-10 ADC	Control LC	WPC-10 LC
Initial BW (g)	70 ± 4 (16)	69 ± 3 (16)	68 ± 7(16)	70 ± 5 (16)	70 ± 5 (18)	70 ± 4 (18)
Final BW (g)	373 ± 25^{ab} (16)	377 ± 27^{a} (16)	371 ± 34^{ab} (16)	373 ± 39^{ab} (16)	347 ± 32^{c} (18)	359 ± 30^{bc} (18)
Total FI (g)	1584 ± 200^{a} (16)	1473 ± 185^{bc} (16)	1563 ± 172^{ab} (16)	1563 ± 195^{ab} (16)	1460 ± 163^{c} (18)	1493 ± 191^{abc} (18)
Feed efficiency (g wt gain/g diet)	0.19 ± 0.02^{b} (16)	0.21 ± 0.03^{a} (16)	0.20 ± 0.03^{b} (16)	0.19 ± 0.02^{b} (16)	0.19 ± 0.02^{b} (18)	0.19 ± 0.02^{b} (18)
3-Day balance during first week						
Apparent Ca absorption (%)	87.3 ± 6.2^{c} (16)	90.0 ± 4.0^{b} (16)	88.9 ± 3.6^{bc} (16)	87.7 ± 3.5^{bc} (16)	98.2 ± 0.8^{a} (18)	97.8 ± 1.2^{a} (18)
4-Day balance during eighth week						
Urinary Ca (4-day, mg)	4.9 ± 3.1^{a} (15)	5.5 ± 3.5^{a} (13)	4.8 ± 1.9^{a} (15)	5.0 ± 2.6^{a} (15)	1.0 ± 0.4^{b} (18)	0.8 ± 0.2^{b} (18)
Apparent Ca absorption (%)	47.1 ± 11.5^{b} (15)	46.4 ± 10.8^{b} (13)	49.5 ± 14.1^{b} (15)	45.6 ± 10.3^{b} (15)	96.8 ± 1.3^{a} (18)	97.7 ± 0.9^{a} (18)
Ca retention rate (%)	45.8 ± 11.4^{b} (15)	44.9 ± 10.6^{b} (13)	48.1 ± 14.4^{b} (15)	44.1 ± 10.4^{b} (15)	96.2 ± 1.3^{a} (18)	97.2 ± 1.0^{a} (18)

[a] Values are mean ± SD (*n*). Values with different superscript letters in a row differ, $P < 0.05$.
[b] BW, body weight; FI, food intake.

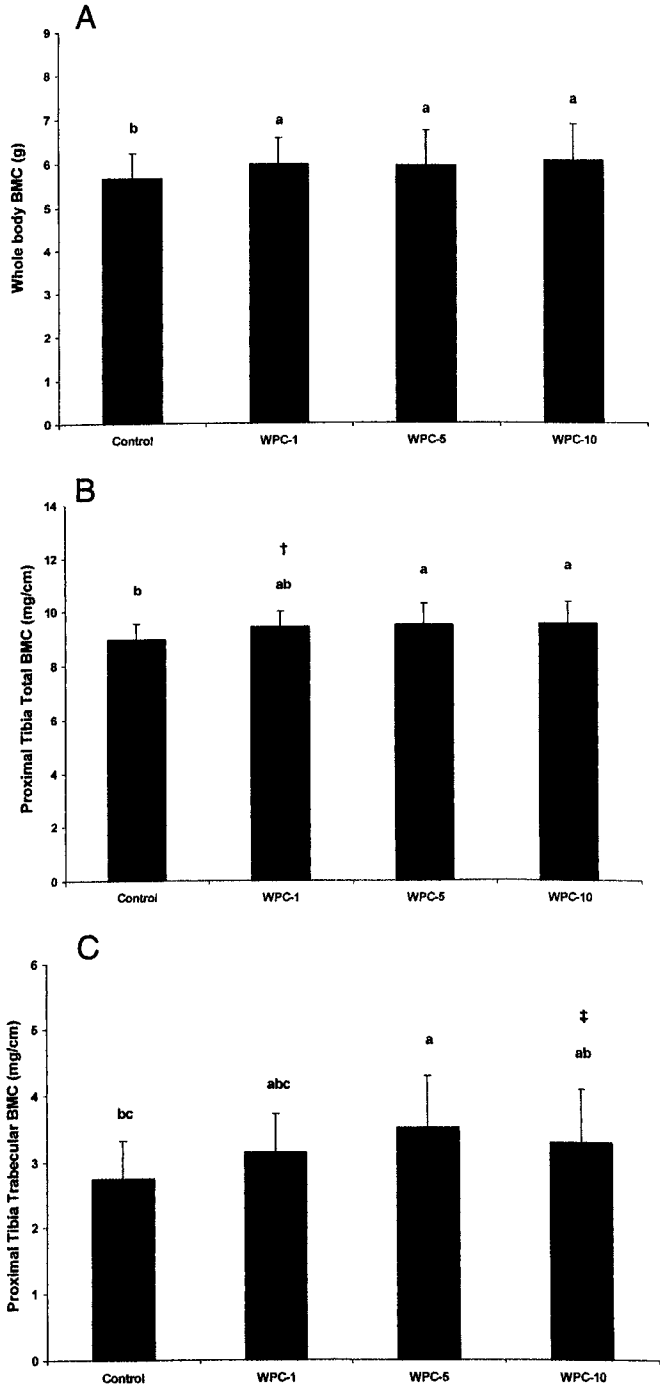

Figure 5. (A) Whole body BMC from DXA among ADC groups after a 7-week feeding ($n = 14$–16/group). (B) Proximal tibia total BMC by pQCT among ADC groups ($n = 11$–12/group). (C) Proximal tibia trabecular BMC by pQCT among ADC groups ($n = 11$–12/group). Values are means ± SD. Different letters indicate significant differences between groups ($P < 0.05$). $^{\dagger}P = 0.077$ compared with ADC control group; $^{\ddagger}P = 0.096$ compared with ADC control group.

Our preliminary studies showed that WPCs increased calcium absorption by 18%–36%. However, this calcium-enhancing effect disappeared with chronic feeding. When rats were chronically fed WPC processed from cheese whey containing GMP (WPC-1) at 1%, apparent calcium absorption was still slightly higher during the first week of feeding than in rats on the control diet (90.0 ± 4.0% vs. 87.3 ±

6.2%). This is in line with the observation that younger rats exhibited higher intestinal calbindin expression (14) and were associated with higher calcium absorption efficiency. Thus, the calcium absorption–enhancing effect of the protein was still apparent, although largely blunted relative to the single meal acute study. The calcium absorption–enhancing effect of the WPC had entirely disappeared by week 8. Subsequent calcium balance and ^{45}Ca kinetics showed no differences between WPC and control groups, suggesting that adaptation to the enhancing effect of WPC on calcium absorption had decreased its impact. This effect would be consistent with down-regulation of active calcium absorption, because the parathyroid hormone–vitamin D axis would be suppressed by the initial increase in calcium absorption during WP chronic feeding.

Adaptation to an early nutritional benefit has been shown for other dietary constituents. A diet containing 5% lactulose increased calcium absorption by 20% when fed in a single meal, whereas it had no effect on calcium absorption after 1 or 2 days of chronic feeding (15). Similarly, vitamin C has been shown to enhance nonheme iron absorption in single meal tests in humans (16), whereas after 5 days of feeding of complete diets, vitamin C had a much less enhancing effect on iron absorption (17).

Whole body BMC increased about 5% in WPC groups, which might result from higher calcium absorption early during the chronic feeding period compared with the control group. However, femur total calcium and femur BMC at the proximal, midshaft, and distal regions did not differ among ADC groups. Peripheral QCT has an advantage over DXA in determining volumetric BMD and differentiating trabecular bone from cortical bone. In the proximal tibia, a trabeculae-rich bone, an increase of total BMC in WPC groups was observed along with an increase of trabecular bone in two WPC groups. At low calcium intake, WPC had no effect on bone or calcium metabolism by any parameter measured, which probably reflects an already maximal use of the limited calcium available.

In conclusion, WPCs enhanced intestinal calcium absorption in acute studies. However, the calcium absorption enhancing effect disappeared with chronic feeding in growing rats. It would be interesting to explore ways of prolonging the effect of WPC on calcium absorption. Although calcium absorption was only temporarily affected by 1% WPC in the diet, there was an increase in whole body BMC supported by increased bone content in the proximal tibia in growing rats. Even short-term enhancement of calcium absorption has an impact on bone mineralization.

The authors thank Stephanie Flin, Carol Spahr, Doug Maish, and Mary Larimore for technical assistance.

1. Pantako OT, Passos M, Desrosiers T, Amiot J. Effect of milk proteins on the absorption of calcium and phosphorus in rats: variations of calcium and phosphorus in the portal vein. Int Dairy J 4:37–58, 1994.
2. Offord Calvin E, Felix R, Tullberg-Reinert H, Ginty F, Barclay D, Muhlbauer R, inventors; Societe des Produits Nestle S.A., assignee. Milk Protein Hydrolysate for Addressing a Bone or Dental Disorder. World Intellectual Property Organization WO 00/49885. August 31, 2000.
3. Takada Y, Kobayashi N, Kato K, Matsuyama H, Yahiro M, Aoe S. Effects of whey protein on calcium and bone metabolism in ovariectomized rats. J Nutr Sci Vitaminol 43:199–210, 1997.
4. Takada Y, Matsuyama H, Kato K, Kobayashi N, Yamamura J, Yahiro M, Aoe S. Milk whey protein enhances the bone breaking force in ovariectomized rats. Nutr Res 17:1709–1720, 1997.
5. Aoe S, Toba Y, Yamamura J, Kawakami H, Yahiro M, Kumegawa M, Itabashi A, Takada Y. Controlled trial of the effects of milk basic protein (MBP) supplementation on bone metabolism in healthy adult women. Biosci Biotechnol Biochem 65:913–918, 2001.
6. Toba Y, Takada Y, Matsuoka Y, Morita Y, Motouri M, Hirai T, Suguri T, Aoe S, Kawakami H, Kumegawa M, Takeuchi A, Itabashi A. Milk basic protein promotes bone formation and suppresses bone resorption in healthy adult men. Biosci Biotechnol Biochem 65:1353–1357, 2001.
7. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951, 1993.
8. Zafar TA, Weaver CM, Zhao Y, Martin BR, Wastney ME. Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. J Nutr 134:399–402, 2004.
9. Hanes DA, Weaver CM, Wastney ME. Calcium and oxalic acid kinetics differ in rats. J Nutr 129:165–169, 1999.
10. Zhang Q. Effects of calcium salt and IGF-I infusion on calcium kinetics and bone parameters in growing rats. PhD thesis, Purdue University, West Lafayette, IN, 2001.
11. Cai J, Zhang Q, Wastney ME, Weaver CM. Calcium bioavailability and kinetics of calcium ascorbate and calcium acetate in rats. Exp Biol Med 229:40–45, 2004.
12. Bennett T, Desmond A, Harrington M, McDonagh D, FitzGerald R, Flynn A, Cashman KD. The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. Br J Nutr 83:673–680, 2000.
13. Younes H, Demigné C, Rémésy C. Acidic fermentation in the cecum increases absorption of calcium and magnesium in the large intestine of the rat. Br J Nutr 75:301–314, 1996.
14. Pansu D, Bellaton C, Bronner F. Developmental changes in the mechanisms of duodenal calcium transport in the rat. Am J Physiol 244:G20–G26, 1983.
15. Brommage R, Binacua C, Antille S, Carrie AL. Intestinal calcium absorption in rats is stimulated by dietary lactulose and other resistant sugars. J Nutr 123:2186–2194, 1993.
16. Cook JD, Monsen ER. Vitamin C, the common cold, and iron absorption. Am J Clin Nutr 30:235–241, 1977.
17. Cook JD, Reddy MB. Effect of ascorbic acid intake on nonheme-iron absorption from a complete diet. Am J Clin Nutr 73:93–98, 2001.